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14. ABSTRACT It has been suggested that PHSCN inhibits metastatic invasion by forming a covalent, disulfide bond with a cysteine residue on the beta1 (β 1) subunit of alpha5 beta1 (α 5 β 1) integrin1. However, these studies were performed with purified α 5 β 1 integrin, which also produces evidence of covalent bond formation with the α 5 subunit (tandem mass spectroscopy data not shown). Hence, the specificity of the reported interaction between PHSCN and the β 1 subunit1 is suspect. Moreover, the cysteine-rich β 1 subunit can heterodimerize with 12 distinct alpha integrin subunits2, forming integrins that function in many pathways. In contrast, the α 5 subunit interacts uniquely with the β 1 subunit2 to induce invasion and support adhesion3-5; hence it is a much more desirable target.					
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INTRODUCTION: It has been suggested that PHSCN inhibits metastatic invasion by forming a covalent, disulfide bond with a cysteine residue on the beta1 ($\beta 1$) subunit of alpha5 beta1 ($\alpha 5 \beta 1$) integrin¹. However, these studies were performed with purified $\alpha 5 \beta 1$ integrin, which also produces evidence of covalent bond formation with the $\alpha 5$ subunit (tandem mass spectroscopy data not shown). Hence, the specificity of the reported interaction between PHSCN and the $\beta 1$ subunit¹ is suspect. Moreover, the cysteine-rich $\beta 1$ subunit can heterodimerize with 12 distinct alpha integrin subunits², forming integrins that function in many pathways. In contrast, the $\alpha 5$ subunit interacts uniquely with the $\beta 1$ subunit² to induce invasion and support adhesion³⁻⁵; hence it is a much more desirable target. Our funded research is designed to test definitively the hypothesis that both PHSCN and PhScN peptides inhibit $\alpha 5 \beta 1$ integrin fibronectin receptor-mediated invasion through noncovalent interaction with $\alpha 5 \beta 1$ receptors of SUM149PT and MDA-MB-231 metastatic human breast cancer cells, and to lay the foundation for detailed structure-activity relationship (SAR) studies for both peptides as inhibitors of $\alpha 5 \beta 1$ -mediated metastatic breast cancer invasion by precisely defining their target site(s) on $\alpha 5 \beta 1$ integrin, and by developing an assay that can be used to evaluate *in vitro* degradation rates in plasma, and to monitor *in vivo* plasma levels.

Objective/Hypothesis: Our preliminary data suggest that the invasion inhibitory potencies of the S-acetylated and the S-methylated PHSCN peptide derivatives are increased by over 1,000-fold, relative to PHSCN. In addition, the PhScN peptide is still more potent: preliminary results suggest that it is 10^4 to 10^5 -fold more potent than PHSCN at blocking $\alpha 5 \beta 1$ -mediated invasion. Thus, we hypothesize that the actual invasion-inhibitory interaction of PHSCN with $\alpha 5 \beta 1$ integrin involves a noncovalent interaction with the target site, which is promoted by including D-isomers of histidine and cysteine in the PhScN peptide. Thus, covalent, disulfide bond formation with $\alpha 5 \beta 1$ integrin is actually a side reaction that *decreases* potency. Because mammalian proteins do not contain D-amino acids, endoproteinses evolved to cleave between L-amino acids only. Since PhScN does not contain 2 linked L-amino acids, it cannot be degraded by endoproteases, usually present at high levels in tumors and increasing with malignancy⁶⁻⁸. Thus, in addition to promoting noncovalent interaction with its $\alpha 5 \beta 1$ integrin target, the inclusion of D-amino acids at alternate positions is expected to result in increased PhScN potency as a result of preventing endoproteolytic degradation. Our research is designed to achieve the following specific aims.

Specific Aims: Specific Aim 1: To lay the foundation for determining their structure-activity relationships (SARs) as inhibitors of $\alpha 5 \beta 1$ -mediated invasion^{3-5,9,10}, we propose to assay PHSCN and PhScN peptide derivatives, having modifications of N- or C-termini, and/or cysteine side chain. Peptides will be tested in quantitative serum-free invasion assays with naturally occurring basement membranes^{3-5,9-15}.

Specific Aim 2: To identify the binding site for the PHSCN sequence on $\alpha 5 \beta 1$ integrin, which will aid in the development of potent PHSCN/PhScN analogs, biotinylated, S-BPM-derivatized PHSCN and PhScN peptides will be photocrosslinked to SUM149PT and MDA-MB-231 cells. Tagged cells and extracts will be screened by fluorescent antibody staining and Western blotting for biotin and $\alpha 5 \beta 1$ integrin. The identity of the BPM-modified subunit and sites(s) of modification will then be determined using tandem mass spectrometry to localize the PHSCN/PhScN binding site(s) on $\alpha 5 \beta 1$ integrin.

Specific Aim 3: To develop a mass spectrometry-based quantitative assay for PHSCN sequence analogs, including PhScN, and use it to evaluate and enhance their stabilities in human and mouse plasma. This assay is crucial for future *in vivo* studies where bioavailability and clearance rates are to be monitored.

BODY:

Specific Aim 1

We proposed to test PHSCN and PhScN derivatives with modifications of the N- or C-termini, and/or cysteine side chain to determine the initial spatial constraints of the binding pocket of the ligand to its target site. This will aid in the selection of crosslinking agents, modifications, tag additions and strategies for determination of

the target site(s) performed in Aim 2 and lay the foundation for determining the structure-activity relationships (SARS) of these inhibitors with the target.

Selection of peptides.

Table 1 is an initial list of peptides for testing and the IC₅₀'s determined using our *in vitro* invasion assay. Initial focus has been on modification of the cysteine side chain and the C-terminus. Modification of the cysteine side chain shows increased potency to the L-cysteine and no change to the D-cysteine activity. The addition of the biotin and the creation of a MAP (multi-antigenic peptide) on the C-terminus and the retention of activity suggest that the “tail” of the peptide has room for modification. These initial results suggest that the C-terminus and cysteine side chain are the logical place to start for placement of cross-linkers for work in Aim 2.

Table 1. Invasion inhibitory potency of PHSCN and PhScN derivatives. Values expressed in Molarity to adjust for molecular weight of modifications. **C**, commercially synthesized; **D**, derivatization in house of the appropriate commercial parent compound; **TBD**, to be determined; **bio**, biotin tag; **MAP**, multiantigenic peptide; **Me**, S-methylated; **OAc**, S-acetylated; **acm**, S-acetamidomethyl; **BPM**, benzophenone-4-maleimide

Table 1	C or D	mw	MDA-MB-231 IC₅₀ M	SUM149PT IC₅₀ M	L or D isomer	Modification Region
Ac-PHSCN-NH ₂	C	598	2.8 E-8	1.5 E-8	L	parent
Ac-PHSCNGGK-bio	C	1180	4.8 E-9	7.3 E-9	L	tail
Ac-PHSC(Me)N-NH ₂	C	612	5.3 E-13	4.0 E-13	L	side
Ac-PHSC(OAc)N-NH ₂	C	640	1.4 E-14	7.3 E-15	L	side
Ac-PHSC(acm)NGGK-bio	C	1537	TBD	TBD	L	Tail + side
Ac- PHSC(BPM)GGK-bio	D	1436	TBD	3.6 E-14	L	Tail + side
Ac-PHSCNGGK-MAP	C	7575	2.0 E-11	2.0 E-11	L	Tail (MAP)
Ac-PhScN-NH ₂	C	598	2.2 E-14	1.3 E-13	D	parent
Ac-PhScNGGK-bio	C	1180	1.3 E-13	4.4 E-14	D	tail
Ac- PhSc(BPM)GGK-bio	D	1436	1.2 E-14	2.5 E-14	D	Tail + side
Ac-PhSc(acm)N-GGK-bio	C	1537	TBD	TBD	D	Tail + side
Ac-PhScNGGK-MAP	C	7575	1.7 E-20	5.8 E-21	D	Tail (MAP)

Derivatization of the peptide ligands with a photoactivatable BPM cross-linker

Under the direction of Dr. Phil Andrews, a protocol for the derivatization of the Ac-PHSCNGGK-bio and the Ac-PhScNGGK-bio with BPM (benzophenone-4-maleimide) was developed. It was determined that the peptide required a pretreatment of TCEP, a phosphine reducing agent, to reduce any dimer formation of the peptide and that the derivatization protocol required a higher concentration of organic solvent to maintain solubility of the cross-linker. Once derivatized the modified peptide is stable and can be cleaned by C18 column as necessary. The modified peptides were analyzed by MS/MS and were found to have acquired the BPM tag, the parent compound was detected but not determined to be significant. The modified peptides were then tested by our *in vitro* invasion assay to determine their potency and are listed in Table 1.

Results of *in vitro* invasion assays for C-terminus and/or cysteine-modified peptide ligands.

Figure 1 shows the invasion inhibitory affects in our standard SU-ECM serum-induced invasion assay for the BPM derivatized compounds that will be used in the crosslinking experiments in Aim 2. Functionally, it can be seen that the bench-top derivatization with BPM (benzophenone-4-maleimide) of the cysteine of PHSCN

produces a more potent compound. This is consistent with commercial preparations of the cysteine derivatives S-Me and S-OAc (Table 1). Derivatization of the D-cysteine with BPM does not affect the IC50. Hence, the modification of the side chain cysteine does not impair the functionality of the compound, and in the case of PHSCN actually improves it. This allows for work in Aim 2 with the BPM compounds for target site determination to begin.

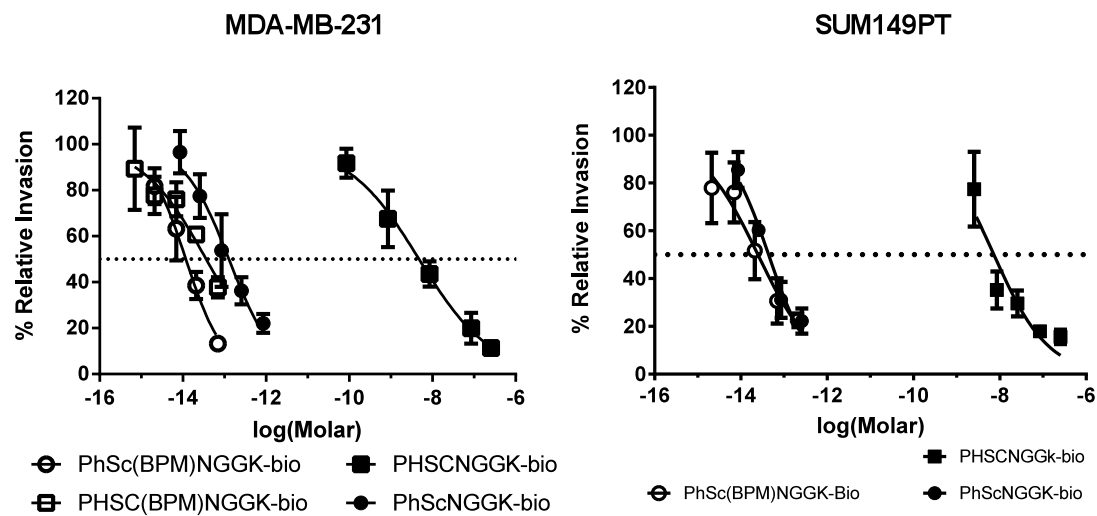


Figure 1. Invasion inhibition with BPM derivatized cysteine of PHSCN and PhScN peptides

Specific Aim 2

Identification of the binding site for the PHSCN/PhScN sequence on $\alpha 5 \beta 1$ integrin using protein crosslinking techniques and subsequent identification of the protein and peptide location by tandem mass spectrometry.

Crosslinking with BPM derivatized peptides and analysis by Western Blotting

PHSCN binds to activated integrin, specifically the $\alpha 5 \beta 1$ integrin. The exact subunit and location is the goal of this proposal. Activation of integrins can be done by divalent cations or by specific ligands. Our first attempt at crosslinking the peptide to its target protein was to replicate the conditions used for *in vitro* invasion assays. Serum starved cells were stimulated by introduction of serum and were then allowed to incubate with the BPM derivatized peptides, followed by UV crosslinking to activate the BPM tag. To simplify and avoid losses, no washing steps were performed. As shown in Figure 2, the primary biotin labeled band is much smaller than the subunits of $\alpha 5 \beta 1$ integrin and their expected mass assuming full glycosylation of the asparagine in the primary sequence (Table 2).

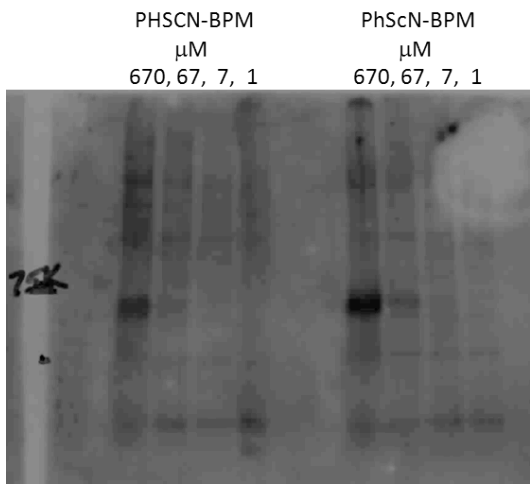


Figure 2. SUM149PT cells, serum starved overnight. Cells suspended into medium at 37 C for 45 minutes. 40,000 cells were incubated with various concentrations of BPM derivatized peptides for another 20 min. UV irradiation for 45 minutes at room temp. 7.5% SDS-PAGE blotted with Anti-biotin.

Table 2	Da Primary sequence	Da fully glycosylated
$\alpha 5$ subunit	114,536	150,964
$\beta 1$ subunit	88,465	119,689

The presence of the major low molecular weight biotin labeled protein suggests that two possible scenarios may have occurred. Either the BPM–peptide has formed an intermolecular crosslink with a nearby protein instead of an intramolecular crosslink within the target protein or the presence of 10% serum introduced a nonspecific binding target. Considering the high concentration of the peptide necessary to observe this low mass complex, the data suggest either 1) nonspecific binding or 2) that the BPM peptides have a very high dissociation constant (K_d). To reduce or eliminate nonspecific binding, further attempts at crosslinking will be performed after integrin activation by divalent cations. To ascertain whether the modified peptides have altered binding properties because of steric hindrances not detected by the functional *in vitro* invasion assay, the binding constants (K_d) of the relevant compounds will be determined.

Crosslinking after activation with Divalent Cations

The crosslinking experiment, whose results are shown in Figure 2, was repeated using divalent cations to activate integrin and a varying amount of cells to increase signal. As shown in Figure 3, the use of divalent cations eliminated the prevalent biotin labeled low molecular weight complex suggesting that this was indeed nonspecific binding to the serum proteins. The presence of bands in the region of interest is promising, however the amount observed suggests that the binding constants of the BPM peptide derivatives are much higher than predicted. The presence of bands only in the L lanes for Ac-PHSCNGGK-bio suggests that there are more steric hindrances in binding for the D lanes, Ac-PhSc(BPM)NGGK-bio than for Ac-PHSC(BPM)GGK-bio.

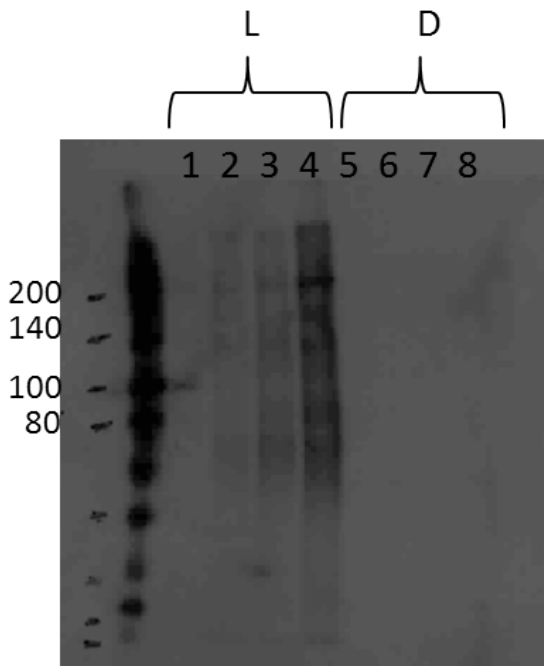


Figure 3. Western blot with Anti-biotin of a 7.5% SDS-PAGE of varying amounts Mn^{2+} activated MDA-MB-231 cells incubated with 50 μM of BPM derivatized peptide. Ac-PHSC(BPM)NGGK-bio(L) and Ac-PhScN(BPM)NGGK-bio(D) denote BPM ligand. The cell quantity varied for each lane in 1,5 50K; in 2,6 100K; in 3,7, 200K and in 4,8, 500K.

Crosslinking with the amine reactive compound DC4.

Our collaborator, Dr. Phil Andrews has developed a cell permeable cross-linker that targets amine groups, called DC4, which spans 15 angstroms. It has also been found to have a minor reaction with histidine and cysteine residues. The lysine in Ac-PhScNGGK-bio has been labeled with biotin and is thereby unavailable for the primary amine reaction of DC4. However the presence of both histidine and cysteine in the sequence allow

for a low level reaction with DC4. As before, cells were activated by divalent cations or FBS, followed by incubated with the biotin labeled peptide, Ac-PhScNGGK-bio. After incubation the cells were quickly spun and the excess peptide was removed, the cell pellet was then resuspended in the DC4 cross-linker. Crosslinking is rapid and complete in about 20-30 minutes. Since any lysine can react, the concentration of the DC4 and the available protein must be empirically determined.

Figure 4 shows the results of the DC4 cross-linker as compared to crosslinking with BPM derivatives. In Panel 4A, it can be seen that DC4 has cross-linked the biotin labeled peptide Ac-PhScNGGK-bio, corresponding to molecular weights of approximately 120 KDa and 140 KDa, which are the mass of the $\alpha 5\beta 1$ integrin subunits. By using DC4, the Ac-PhScNGGK-bio is able to bind at its normal binding K_d without steric interference, and the DC4 can react with either the histidine or the cysteine. Unlike reaction with lysine, reaction to histidine or cysteine is a reversible by using hydroxylamine. The ability to reverse and confirm this type of reaction may be useful in identifying the target site peptides with MS/MS.

In Panel B, the L and D BPM derivatives behave differently, again suggesting steric interference of the BPM group. However, it is interesting to note that the labeled bands are of similar molecular weight as those in Panel A. This is encouraging and attempts to scale the experiment to levels where enrichment and protein detection can be observed with staining will continue.

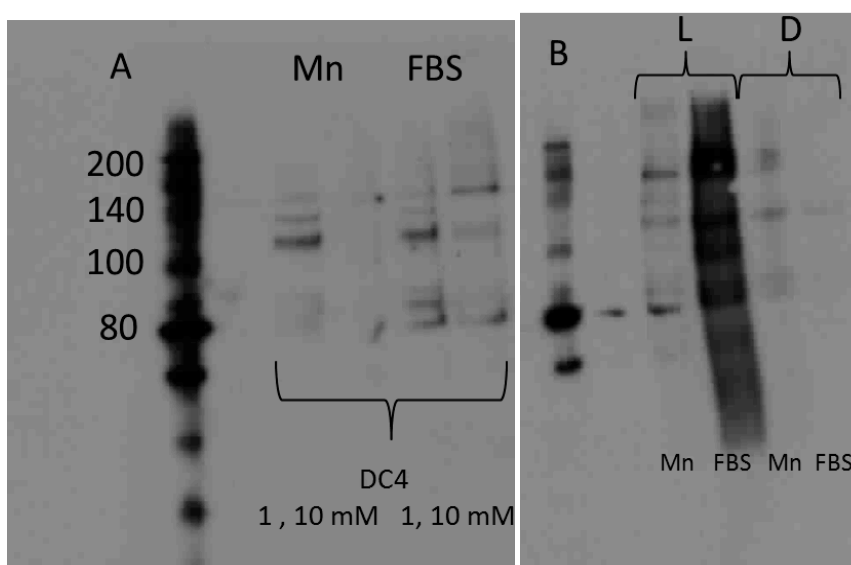


Figure 4. Western blot with Anti-biotin primary antibody, of a 7.5% SDS-PAGE of activated SUM149PT cells using DC4 or the BPM peptides. The 100 K cells were activated using either divalent cation(Mn^{2+}) or FBS. Panel A) The concentration of 100 μM Ac-PhScNGGK-bio was used, cells were spun to remove excess labeled peptide prior to resuspension in DC4 crosslinker. Panel B) 200 μM Ac-PHSC(BPM)NGGK-bio(L) and Ac-PhScN(BPM)NGGK-bio(D) were incubated with cells and then subjected to UV light. The samples were prepared at the same time and run on the same gel and developed together. However, different exposures were required to optimize each panel. Images were scanned and then overlaid.

The cross-linking with the BPM peptides will continue; attempts will be made to scale the experiments for protein enrichment and identification by MS/MS. The DC4 cross-linker will complement this work, and will also continue. Preliminary evidence indicates that there are steric hindrances by modification of the cysteine,

therefore a new peptide, Ac-PhScNGGK(biotin)GK-NH₂, will be synthesized to allow for cross-linking via the C-terminal lysine residue with either DC4 or other commercially available amine reactive cross-linking agents.

Determination of Binding Constants

The presence of a biotin tag on the peptide allows for a direct determination of the binding constant (K_d) to live cells by detection with streptavidin-HRP. In Figure 5, the K_d of the Ac-PhScNGGK-bio was determined and compared to Ac-PHSCNGGK-bio; they were found to be similar, at approximately 0.03 μ M for both MDA-MB-231 and SUM149PT cells. The K_d determination for the BPM-derivatized biotin peptides is in progress and will provide information on any steric hindrances with the BPM cysteine modification not detected in the functional assay.

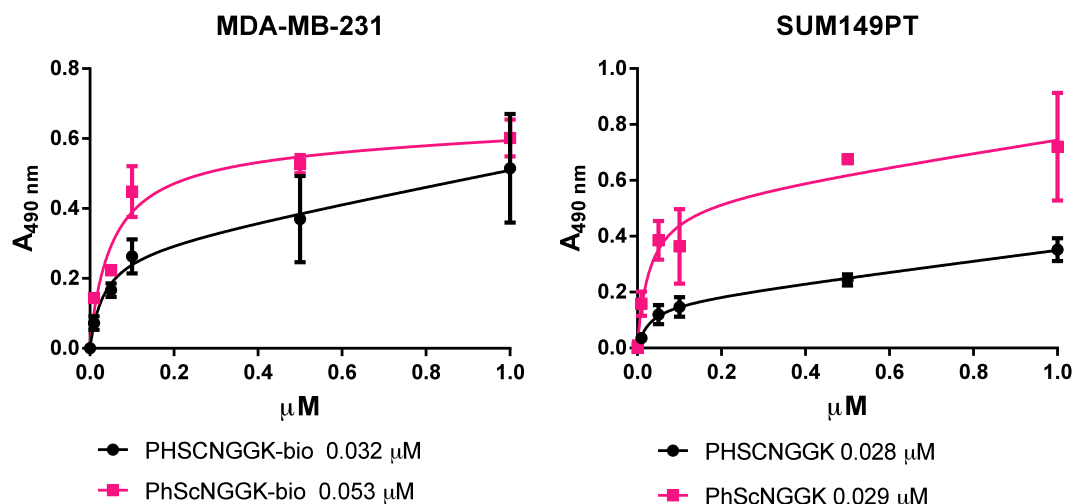


Figure 5. Determination of the K_d values for the underivatized biotinylated peptides with suspended MDA-MB-231 or SUM149PT cells.

Binding characteristics of non-biotin labeled peptides can be assessed indirectly by competition binding assays. These assays require a constant concentration of labeled peptide near its K_d and is dissociated by increasing amounts of an unlabeled competitor. This generates an inhibition constant (K_i) which will allow comparison of binding properties of the unlabeled peptides. This assay will also confirm whether ligands are competing for the same binding site. Figure 6 shows the competition assay for the parent compounds PHSCN and PhScN and confirms that the peptides compete for the same site. These binding assays in conjunction with the functional assay results in Table 1 will allow a more complete profile of the peptide ligand properties

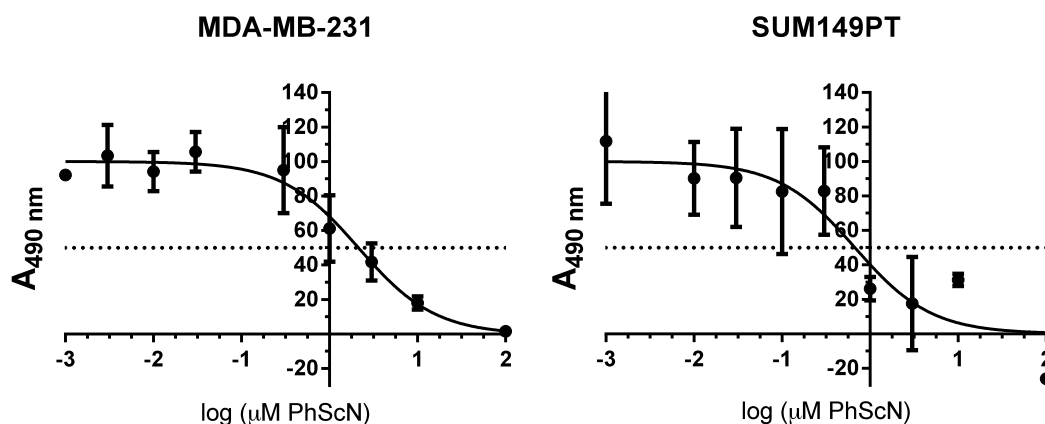


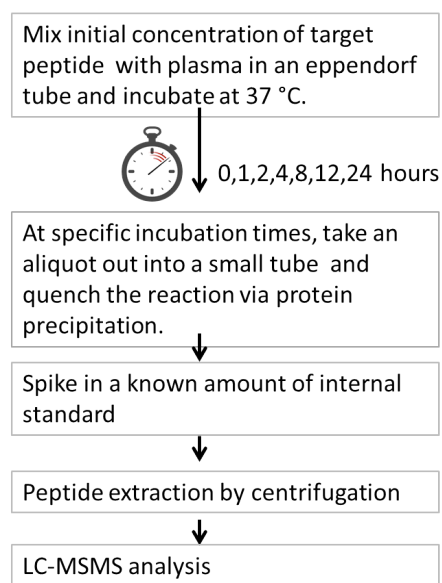
Figure 6. Competition binding assays: Ac-PHSCNGGK-bio and dissociation with unlabeled Ac-PhScN-NH₂.

Specific Aim 3

Develop a mass spectrometry-based quantitative assay for PHSCN analogs, including PhScN, and evaluate their stabilities in human and mouse plasma.

Experimental design for quantitative assay of PHSCN analogs

An experimental approach based on mass spectrometric analysis to evaluate the stabilities of PHSCN analogs in human and mouse plasma has been designed as outlined in Scheme 1. A known amount of target peptide is mixed with plasma and incubated at 37 °C for up to 24 hours. At specific incubation time points an aliquot is transferred to a small tube and protease reactions were quenched by adding three volumes of 50% acetonitrile with 0.5%TFA. Precipitated plasma proteins were removed by centrifugations, and a known amount of an internal standard was spiked into the supernatant containing the peptides, in order to increase the accuracy of quantitative measurements of LC-MS by reducing variances associated with sample preparations. MRFA (Met-Arg-Phe-Ala, monoisotopic molecular mass = 523.247.), a stable synthetic peptide with similar molecular mass to the target peptide, Ac-PHSCN-amide (monoisotopic molecular mass = 597.2328), is added into samples prior to LC-MS analysis. Stabilities of target peptides in plasma were evaluated by LC-MS.



Scheme 1. Experimental workflow

Mass spectrometry

The LTQ-Orbitrap XL(Thermo Finnegan) connected to a 2D nanoLC(Exigent) and an autosampler in Andrews laboratory provides high mass resolution and accuracy for analytes separated on a capillary column at a flow rate of 200 nL/min, which assures confident identification of target molecules. Extracted peptides from plasma are reconstituted in 0.1% TFA and loaded into a C18 trap by autosampler and washed for 6 min at 5 microL/min with 0.1% TFA in water delivered by the LC loading pumps. Trapped peptides were introduced by switching valve into a capillary column (75 micro-m x 15 cm) custom-packed with C18 resin (3 micron) and

separated over 60 min gradient at a flow rate of 200 nL/min. Analytes were introduced into the LTQ-Orbitrap via an electrospray device (Triversa Nanomate, Advion). MS and MSMS data were acquired in data-dependent mode for ions within 400-800 m/z. Figure 7 shows the total ion chromatogram (TIC) of LC-MSMS analysis of PHSCN and the internal standard MRFA.

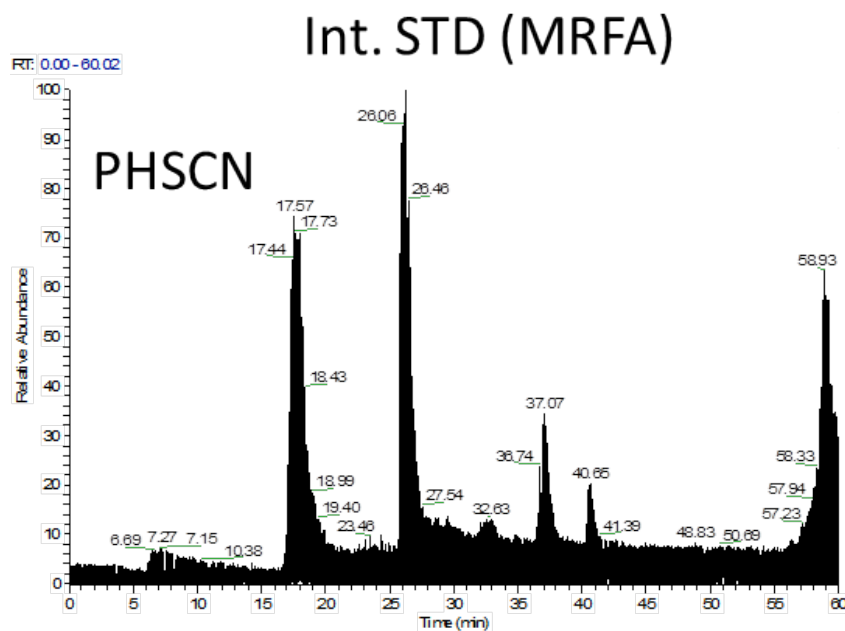


Figure 7. TIC from an LC-MSMS of PHSCN and MRFA mixture.

Quantitative assay of target peptides

Peptide quantification can be achieved by ion intensities in MS or by MSMS spectral counts in data-dependent acquisition mode. These two approaches are well established label-free quantification methods for peptides and proteins by LC-MSMS, which can be readily applied to a bioanalytical assay of therapeutic peptides. We are currently testing a new assay method, an MRM-like analysis via data-independent acquisition mode in the LTQ-Orbitrap, in which ion packets within a mass range of 5 or 10 Da accumulated for a set time are co-fragmented. One or multiple fragment ions specific to the target peptide can be monitored in MSMS based on highly accurate precursor ion mass and LC retention time. This MRM-like approach can provide a highly selective and sensitive bioanalytical assay under conditions of high chromatographic interference.

Study of collision-induced dissociation (CID) fragmentation of Ac-PHSCN-NH₂ in online LC-LTQ-Orbitrap

Ac-PHSCN-NH₂ introduced into the mass spectrometer via a nanoESI device (Triversa Nanomate) was predominantly observed as singly protonated form at 598.2328 m/z. However, in online LC-MS, the peptide was observed in doubly charged dimeric forms at 597.23 m/z and 589.23 m/z. The peptide dimer ions were characterized by CID that revealed disulfide-bond formation between two peptides that was catalyzed on C18 LC, as shown in a CID spectrum of 597.23 m/z. This is a potential source of interference with the assay that can be easily addressed by incorporating reduction and alkylation of the Cys residues into the protocol prior to LCMSMS.

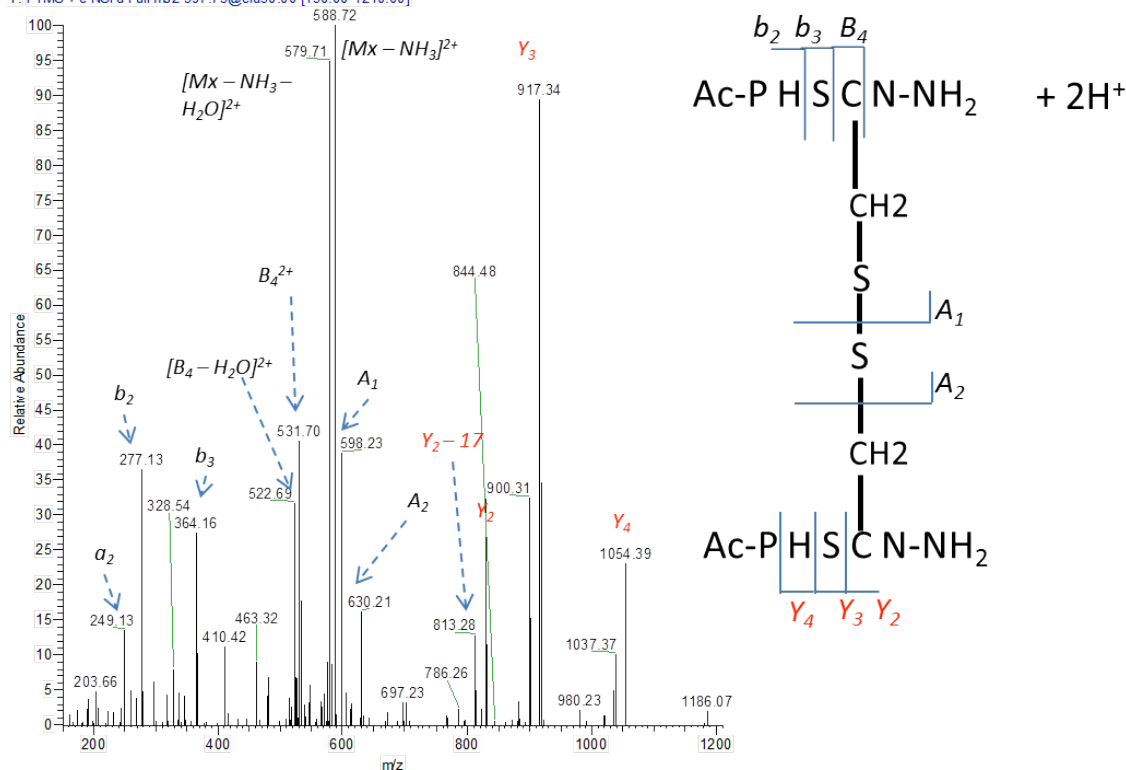


Figure 8. CID fragmentation of doubly-charged ion at 597.73 m/z (the second isotope to 597.23 m/z). Fragment ions B4, A2, Y2, Y3, and Y4 containing S-S cross-linked peptides confirm disulfide bond formation between two thiols of cysteine.

KEY RESEARCH ACCOMPLISHMENTS:

1. Determination of invasion-inhibitory potencies for a total of 12 PHSCN and PhScN derivatives, targeting $\alpha 5 \beta 1$ integrin-mediated invasion of naturally serum-free basement membranes by 2 metastatic human breast cancer cell lines, SUM149PT and MDA-MB-231. Demonstration that covalent S-modification by several different moieties increases invasion-inhibitory potency by 100,000- to 1,000,000-fold, and that a 1,000,000-fold increase in potency is also obtained by substitution of D-His and D-Cys. These results demonstrate that the reason for the greatly increased invasion-inhibitory potency of PhScN is the suppression of covalent disulfide bond formation between the PHSCN sequence and its integrin target, and hence they are a key step in the determination of the PhScN target site on $\alpha 5 \beta 1$ integrin receptors of breast cancer cells.
2. Determination of invasion-inhibitory potencies of PHSCN and PhScN peptides, containing BPM-derivatized cysteine or D-cysteine. The greatly increased invasion-inhibitory potencies of the BPM-modified PHSCN and PhScN peptides also confirm the noncovalent nature of invasion inhibition by the PhScN peptide.

Research accomplishments 1 and 2 complete Specific Aim 1 of the SOW.

3. Crosslinking with BPM-derivatized peptides, after integrin $\alpha 5 \beta 1$ activation with divalent cations, and analysis by Western blotting. Successful crosslinking with biotin labeled peptides, modified either at the cysteine or on the C-terminus, is anticipated to pave the way for protein enrichment and binding site identification by MS/MS.
4. Determination of binding constants for PHSCNGGK-Bio and PhScNGGK-Bio on MDA-MB-231 and SUM149PT cells, and demonstration of their similarity provides the basis for acquiring information on any

steric hindrances introduced by modification of the ligands not detected in functional invasion assays.

5. Demonstration that PHSCNGGK-Bio and PhScNGGK-Bio compete for the same binding site on SUM149PT and MDA-MB-231 cells. The results of these binding assays, in conjunction with the functional invasion inhibition results shown in Table 1, will allow a more complete profile of the peptide ligand properties and their functional effects on $\alpha 5 \beta 1$ integrin-mediated invasion. Moreover, these results provide key support of the hypothesis that the increased potency of the PhScN peptide results from suppression of covalent, disulfide bond formation with the integrin target.

Research accomplishments 3, 4, and 5 provide key support towards accomplishing Specific Aim 2 of the SOW.

6. Demonstration of CID fragmentation of Ac-PHSCN-NH₂ in online LC-LTQ-Orbitrap indicates that development of a mass spectrometry-based quantitative assay for PHSCN analogs, including PhScN, for the purpose of evaluating their stabilities in human and mouse plasma (Specific Aim 3) is underway.

REPORTABLE OUTCOMES: None to date.

CONCLUSION: The PhScN peptide is a highly potent inhibitor of $\alpha 5 \beta 1$ integrin-mediated, serum-induced invasion because it suppresses covalent, disulfide bond formation with its target. Moreover, results of competition assays show that it targets the same region of $\alpha 5 \beta 1$ integrin as the PHSCN peptide parent.

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